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REVIEW ARTICLE

Stem Cells Derived from Amniotic Fluid: A Potential Pluripotent-Like Cell Source for Cellular Therapy?

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Abstract: ***Background:*** Regenerative medicine aims to provide therapeutic treatment for disease or injury, and cell-based therapy is a newer therapeutic approach different from conventional medicine. Ethical issues that rose by the utilisation of human embryonic stem cells (hESC) and the limited capacity of adult stem cells, however, hinder the application of these stem cells in regenerative medicine. Recently, isolation and characterisation of c-kit positive cells from human amniotic fluid, which possess intermediate characteristics between hESCs and adult stem cells, provided a new approach towards realising their promise for fetal and adult regenerative medicine. Despite the number of studies that have been initiated to characterize their molecular signature, research on developing approaches to maintain and enhance their regenerative potential is urgently needed and must be developed.

Aim: Thus, this review is focused on understanding their potential uses and factors influencing their pluripotent status *in vitro*.

Conclusion: In short, this cell source could be an ideal cellular resource for pluripotent cells for potential applications in allogeneic cellular replacement therapies, fetal tissue engineering, pharmaceutical screening, and in disease modelling.

Keywords: Amniotic fluid stem cells, pluripotency, fetal stem cells, regenerative medicine.

1. INTRODUCTION

Regenerative medicine aims to repair and regenerate cells, tissues or organs to restore compromised function due to diseases, tissue loss, ageing and injury. Currently, organ transplantation is the treatment employed for end-stage diseases or massive tissue loss [1]. However, such interventions are challenging due to long waiting lists, limited donor availability, low immunocompatibility of donor and recipient tissues which necessitates for lifelong immunosuppression, and causes complications due to graft-versus-host disease [2, 3]. Therefore, stem cell technologies are expected to compensate for the therapeutic limitations of reconstructive surgery and organ transplantation. Stem cell-based therapy could be useful to cure multiple inherited and degenerative disorders, owing to their unique properties, such as self-renewal and differentiation potential which serves as a resource that allows to have the required number of therapeutically relevant cell types available [4]. As such, amniotic fluid (AF) has attracted increasing attention as an excellent source of stem cells for regenerative medicine, since it harbours stem cell populations that are high in plasticity to self-renew and differentiate into multiple lineages, without the risk of raising ethical controversies that are typically encountered with other sources [5-7]. Particularly, as AF is usually collected during the second trimester for genetic prenatal screening, it has long been considered as medical waste, thus enabling AF to be easily accessible and abundantly available. Because of the high therapeutic potential of amniotic fluid-derived stem cells (AFSCs), they may thus could serve as a valuable tool for cell therapy and tissue engineering applications.

Nonetheless, further investigations are required to understand whether AFSCs would really represent an intermediate cell type with advantages over both embryonic and adult stem cells. The approach to generate clonal AFSC lines as newer tools to investigate their molecular and cellular biological signature profiles would certainly provide a better understanding of their pluripotent-like potential, as well as their utility for disease modelling. In this review, we will discuss the potential of these novel AFSCs in regenerative medicine and their future potential and perspectives.

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Amniotic Fluid-Derived Stem Cells (AFSCS)

AF, a clear fluid surrounding the developing fetus, was first isolated and studied during the beginning of the 20th century [8]. Initial studies focused on using the AF for determining fetal health, and also to characterize the cells in AF [9, 10]. The characterization showed that 2% of the AF within the amniotic cavity is made up of different cell types of fetal origin [11]. The AF contains nutrients and growth factors that facilitate fetal growth, provide mechanical cushioning and antimicrobial effectors that protect the fetus, and further allow the assessment of fetal health [12]. Interestingly, the cellular composition of AF changes during pregnancy, as the embryo develops. During the first trimester, cells within the AF are likely to be originated from the fetal skin and amniotic membrane. After the 16th week of gestation, when fetal lung and kidney function begins, cells are also believed to be released during fetal micturition, from the respiratory and intestinal tracts [13].

Recently, there are numerous reports on cells present in the AF. Specifically, a population of cells existing within the AF is heterogeneous, consisting of both specialised and unspecialised cell types. Based on their morphology and growth characteristics, viable adherent cells from AF are classified into three groups - amniotic fluid type (AF-type; approximately 60%), epithelioid (E-type; approximately 34%) and fibroblastic type (F-type; approximately 6%) [14]. The percentage allocations for each cell type and their respective morphological features have been summarized in Table **1**. Specifically, AF-type cells are the most abundant cell source from the AF. These cells are subtly distinct from F-type as they appear less polar and show greater marginal ruffling. The average growth potential of AF-type cells is inferior to those of F-type, but their initial growth rates and tolerance for multiple passaging make it possible to harvest relatively large numbers of these cells within 6 weeks. By contrast, E-type cells are large polygonal cells with smooth margins and have *in vitro* growth potential lower than that of AF- or F-type cells. It is worth noting that the majority of cells present in the AF is terminally differentiated, and thus they have limited proliferative capacity [5, 15]. Nonetheless, intriguingly, it has recently been shown that AF also contains a small population of cells that possess the capacity to differentiate into a wide range of lineages, potentially to all the three primary germ layers, namely the ectoderm, endoderm and mesoderm [16].

2. CHARACTERISTICS OF AFSCs

Cell-based therapy demands large number of cells possessing the potential to restore lost function, enhance repair and regeneration and suppress the disease-related complications. Among the various stem cell sources, stem cells isolated from the inner cell mass of human pre-implantation blastocyst, termed as the human embryonic stem cells (hESCs) hold the highest potential, as they are pluripotent and can differentiate into any cell types belonging to the three germ layers - ectoderm, endoderm and mesoderm [17]. However, the use of hESCs raises ethical issues on the destruction of human embryos, in addition to safety concerning the formation of teratomas *in vivo* [18, 19]. On the other hand, adult stem cells, which are relatively easier to obtain, do not raise ethical concerns regarding their isolation, but they have comparatively lower proliferative and differentiation capacity [20, 21]. This makes them a less ideal choice for cell therapy, since it demands for a large number of cells with higher differentiation potential [22]. Furthermore, adult stem cells from aged donors are usually less potent than those from younger donors, due to the age-related epigenetic and cellular signature changes [23]. Ideally, cell therapy requires stem cells endowed with either pluripotency or broadly having multipotent characteristics, which confer them the potential cells closer to that of pluripotent stem cells, while not being tumorigenic and without any ethical concerns. Thus, initiatives have now stepped up on identifying alternative cells sources with potential that highly resembles the pluripotent stem cells, and which compensates for the ethical concerns and the risk of teratoma formation associated with the use of hESCs. The successful identification of small populations of cells in AF expressing Oct4 and with high proliferative capacity has generated a lot of interest in studying this novel source of stem cells [24]. This has made it possible to harvest stem cells which possess pluripotent characteristics and at the same time are free from ethical constraints.

Isolation of AFSCs

Essentially, the isolation of AFSCs is not limited to AF samples at specific week of gestation [7]. The ideal gestation week where AFSCs can be isolated from the AF is the first trimester, as these cells are generally at a higher primitive state, thereby resembling more of the ESCs. Nonetheless, the downside with isolation of AFSCs from early trimester is that it does not allow for their broader applications in the clinical setting. This resorts to isolating these cells at mid- or late-trimester for most studies investigating the therapeutic characteristics of AFSCs, where they are reported to be still therapeutically fit for use in cell-based therapy [25, 26].

Early studies on AF cells relied on morphological observation of heterogeneous mixture of cells that were found in suspension culture in AF [13]. Later on, cells that adhered to the plastic surface of a culture dish were propagated for fur-

Table 1. Amniotic fluid cell types.

Cell type	Morphology	Percentage	References
Amniotic fluid (AF-type)	Abundant, Less polar, marginal ruffling, high growth rate	60%	(Hoehn *et al.*, 1975)
Epitheliod (E-type)	Large polygonal cells, smooth margins, moderate growth rate	34%	
Fibroblastic (F-type)	Fibroblastic, spindle shape, highest growth rate	6%	

Table 2. Comparison of ES cells, iPS cells, AFSC and MSC.

	ES Cells	iPS Cells	AFSC	MSC
Source	Inner cell mass of preimplantation embryo	Somatic cells	Amniotic fluid	Adult tissues
Markers	Oct3/4, Sox2, Nanog, SSEA4, SSEA3, Tra-1-60, Tra-1-81, ALP	Oct3/4, Sox2, Nanog, SSEA4, SSEA3, Tra-1-60, Tra-1-81, ALP	Oct4, SSEA4, c-kit	CD44, CD73, CD90 and CD105
Pluripotency	Pluripotent	Pluripotent	Broadly multipotent or limited pluripotent	Multipotent
Teratoma	Yes	Yes	No	No
Differentiation	Endoderm, mesoderm, ectoderm	Endoderm, mesoderm, ectoderm	Endoderm, mesoderm, ectoderm	Mesoderm Other lineages (via transdifferentiation)
Immunogenicity	Yes	Yes	No	No
Ethical issues	Yes	No	No	No

ther analysis and were termed AF-MSCs [27]. Particularly, these cells were from AF samples derived from second trimester and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS). Subsequently, identification of Oct4-positive subpopulation of AFSCs [24] led to a more selective method of isolation. This raised the question of whether these cells are pluripotent or they show a partial pluripotent marker expression that confers higher regenerative potential than MSCs from other sources? The immunoselection method came afterwards where cells are selected for the expression of the surface marker CD117 (c-Kit), which accounts for approximately 1% of the total AF cell population [28]. Since then, AFSCs were selected initially on plastic adherence and by discarding the non-adherent floating cells. The "plastic adherence" method is the method where most studies are based on the isolation of AFSCs to date [7]. These adherent cells are further subjected to immunoselection for the cell surface marker, CD117 (c-Kit), through either the fluorescence- or magnetic-activated cell sorting (FACS or MACS) method. These cells were termed as c-Kit+ AFSCs and were also found to be Oct4-positive [16, 28].

It is interesting to note that a two-stage culture protocol has also been established to isolate AFSCs [29]. This isolation protocol differs from the "plastic adherence and immunoselection" method where the former uses non-adhering AF cells. The first stage of this protocol involves collecting the non-adherent cells from culture supernatant derived from AF cells that have been cultured in serum-free media. This is followed by plating these non-adherent cells to grow AFSCs, after analysis of the foetal chromosomes. Besides that, c-Kit+ AFSCs can be directly isolated from AF samples [30]. This method falls short in that it is more effective when the amount of AF sample is large, which is not the case after amniocentesis, where AF that can be collected is rather limited. Nonetheless, under the circumstances when there is amniodrainage or AF is collected at delivery, this direct isolation method can be useful, as large AF sample can, in turn, incapacitate the "plastic adherence and immunoselection" method. Notably, it has also been reported that AFSCs can be isolated using merely the culture condition [31]. This culture condition can be established using high serum-containing media such as DMEM, supplemented with 10-20% FBS and with or without the basic fibroblast growth factor. Cells cultured in this condition have the capacity to differentiate into adipocytes, chondrocytes and osteocytes, as well as myocytes, thus displaying the mesodermal differentiation potential. Overall, it should be highlighted that the variations in terms of the therapeutic potential of AFSCs isolated from the selection (plastic adherence and c-Kit+) method, and the culture conditions alone do exist. The latter produces AFSCs with a purely mesenchymal phenotype [31], while AFSCs with intermediate characteristics between ESCs and MSCs can be obtained with the former method [28]. This clearly demonstrates the significance of isolation protocol in harboring AFSCs with high therapeutic potential. The present review focuses on c-Kit+ AFSCs.

C-Kit+ AFSCs and Their Molecular Signatures

It has been well documented that AF contains a heterogeneous mixture of cells, consisting of both differentiated and undifferentiated types of cells. The two distinct cell populations of interest within the AF are the amniotic fluid mesenchymal stem cells (AFMSCs) and the c-Kit+ amniotic fluid stem cells (AFSCs). Of the two cell populations, AFSCs are more remarkable due to the expression of pluripotency markers and higher differentiation potential, making them good candidates for therapeutic applications [28]. While acquiring knowledge on the molecular signatures of AFSCs is crucial, it is equally important to grasp the timeline where AFSCs were discovered, and continued to be investigated to-date.

Dating back to 2003, Prusa *et al.* demonstrated for the first time the presence of a subpopulation of cells in human AF that expressed the transcription factor Oct4 at both mRNA and protein levels [24]. As Oct4 is known to be responsible for the maintenance of the undifferentiated state and pluripotency of embryonic stem cells and germ cells [32, 33], and thus this evidence strongly implied the possible existence of pluripotent cells within the AF. On the other hand, Scherjon and group revealed another facet of AF cells by

reporting the presence of a population among these cells that expressed markers signifying mesenchymal origin, including CD73, CD90, CD105 and CD166, while haematopoietic markers CD14, CD34 and CD45 were found to be absent in these cell populations [34]. A more thorough elucidation of the phenotypic characteristics of AF cells was brought upon when populations of AF cells positive for CD117 (c-Kit+) were isolated [28]. Essentially, c-Kit is the type III tyrosine kinase receptor of the stem cell factor [35]. As this surface antigen has been found on hESCs [36], primordial germ cells and many adult stem cells, including, but not limited to, those of the neural crest [37, 38], and thus it is regarded as the common marker of stem cells that have multi-potent capacity.

The c-Kit+ AFSCs were found to have higher proliferative capacity with doubling time of 36 hours and grew more readily in a monolayer culture system than the adult stem cells [28]. Furthermore, these AFSCs could be maintained in culture for over 250 population doublings (PDs) with a stable karyotype and long telomeres with an average length of about 20kbp between 20 and 250 PDs. This clearly indicates that AFSCs remain genetically stable in culture, along with evidence that showcased the retention of homogenous, diploid DNA content at G1 phase even after expansion of these cells up to 250 PDs, with no obvious chromosomal rearrangements [28]. Notably, the absence of senescence over the long culture period [28] especially gives AFSCs the advantage over somatic cells and other stem cell types, as the latter two are subject to Hayflick limit [39], thus limiting their proliferative capacity both *in vivo* and *in vitro*. Intriguingly, AFSCs have been found to be non-tumorigenic *in vivo*, which could be an attractive feature for their clinical application [28]. *In vitro* differentiation studies, on the other hand, have found that AFSCs are able to differentiate into cell types representing each germ layer (ectoderm, mesoderm and endoderm), including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages [28]. Other studies reported similar results that have demonstrated the capacity of AFSCs to differentiate into chondrocytes, adipocytes, osteocytes, neurons, haematopoietic and renal cells [29, 34, 40-44]. This broad-spectrum differentiation ability of AFSCs is a desirable trait for providing unlimited source of cells for various clinical applications.

Remarkably, AFSCs have been shown to express some of the hESC markers of pluripotency, such as Oct4, Nanog, Sox2, Klf4, SSEA-4 and c-MYC in numerous studies [16, 28, 45]. In fact, more than 90% of the c-Kit+ AFSCs expressed Oct4, further consolidating the presence of pluripotent stem cells in human amniotic fluid [28]. The expression of other pluripotency markers of ES and EG cells such as SSEA-3, Tra-1-60 and Tra-1-81 varied among different studies, whereby one demonstrated the presence of these surface antigens on about 60% of the cells [16], while another showed the absence of SSEA-3 and Tra-1-81 in AFSCs [28]. The discrepancies in terms of expression of pluripotent markers in AFSCs could be attributed to variations among the AF samples (gestation week) and adaptation of AFSCs in selective culture conditions, which will be discussed later. The pluripotent-like characteristics of AFSCs have been further demonstrated by Valli *et al*. who showed for the first time the ability of AFSCs to form the three-dimensional aggregates called embryoid body (EB) when cultured in suspension [46]. The same outcome has been previously shown when ESCs were grown under the same condition, that is, in the absence of anti-differentiation factors and the lack of contact with feeder cells. Notably, this phenomenon recapitulates the first steps of early mammalian embryogenesis.

Besides displaying the pluripotent-like characteristics, AFSCs expressed a panel of mesenchymal and/or neural stem cell markers such as CD29, CD44, CD73, CD90 and CD105 [28, 29, 47, 48]. AFSCs have also been found to express mesenchymal and epithelial cell markers such as SH2, CD44 and cytokeratin AE1/3, as well as Nestin, tubulin beta 3 and glial fibrillary acidic protein, all of which are neuronal markers [40]. With respect to the above findings, it can be inferred that with characteristics of MSCs while resembling ESCs, AFSCs may possess higher regenerative potential than MSCs. Adding in to the molecular profile of AFSCs, while showing negative results for the major histocompatibility Class II antigen, HLA-DR and AFSCs also did not express the hematopoietic lineage marker (CD45), hematopoietic stem cell markers (CD34 and CD133) and endothelial cell markers (CD14 and CD31) [6, 16, 28, 29]. Taken together, one could conclude that AFSCs are not hematopoietic-related stem cells and they have low immunogenicity. The latter further strengthens their credibility as the stem cell source of higher preference compared to ESCs and MSCs for transplantation medicine.

Having knowing the characteristics of AFSCs from various published studies, it is equally important to conclude on where they stand in the pluripotency hierarchy. In this regard, it is evident that they possess intermediate characteristics between the pluripotent ESCs and the lineage-restricted adult stem cells. Specifically, AFSCs are capable of multi-lineage differentiation, in which these lineages include those representing the three embryonic germ layers, namely the endoderm, ectoderm and mesoderm [28], thus conferring them with higher differentiation capacity than the adult stem cells. This, taken together with the fact that they are non-tumorigenic, unlike ES cells, makes them an even more desirable stem cell source for transplantation. Additionally, as AFSCs can be routinely obtained from backup amniocentesis samples that would otherwise be discarded [28], and thus their isolation does not engage into any ethical controversy like the ESCs. Owing to the facts that AFSCs do not induce tumour *in vivo* while having broad-spectrum differentiation capacity, hence they are often considered as being "broadly multi-potent" or "pluripotent-like". The overlapping molecular signatures between ESCs, MSCs and AFSCs have been summarized in Table **2**.

3. FACTORS INFLUENCING THE PLURIPOTENT-LIKE CHARACTERISTICS IN hAFSCs

3.1. Pluripotent-like Status of AFSCs is Gestational Week-dependent

Pluripotency is defined as the ability to self-renew indefinitely and to differentiate into all cell types in the body [49]. Therefore, high pluripotency is one of the important criteria for choosing stem cell source for cell therapy. Expression of the pluripotency-governing genes in AFSCs

could be influenced by various factors including gestation week, epigenetic status and *in vitro* culture conditions as mentioned earlier [50]. Therefore, identifying the gestational period at which large amount of pluripotent-like pool of c-Kit+ cells can be obtained, is crucial. In a total AF cell sample, pluripotent, mesenchymal and progenitor markers have been documented to be expressed in a time-dependent manner (gestational week) [25, 26]. Pluripotent markers, Oct4 and c-Kit were reported to be expressed throughout the gestation period tested (week 15-20). Furthermore, cells from AF of early gestational period expressed higher levels of mesodermal and endodermal markers which tend to decrease AF harvested from later gestational period. By contrast, ectodermal markers were present consistently in all samples throughout the gestation period. The expression of organ-specific markers also increases with time, concomitantly with organogenesis and fetal development [6].

Studies also showed that, c-Kit+ cells obtained from early gestational period (week 10-12) contain cells that are highly pluripotent, indicated by the expression of Oct4, Sox2, Nanog, Klf4, c-Myc, SSEA4, alkaline phosphatase (AP), Tra-1-60 and Tra-1-81. When cultured in suspension, these cells readily formed EB that expressed markers representative of all three germ layers, indicating their highest differentiation potential [46]. Intriguingly, they failed to generate teratoma upon injection into immunodeficient mice for up to 12 weeks after injection [16]. This may be due to the lower level of pluripotent status than hESCs, and thus may not be sufficient enough to have the ability to form tumor, which is a good trait for transplantation medicine.

Most of the studies using second trimester (week 13-24) AFSCs have been reported showing the expression of the pluripotent marker Oct4, with some reports showing co-expression of Sox2, Nanog, c-myc, Klf4 and SSEA4. Oct4 expression level has been demonstrated to be high between gestation week 15-20 [6, 24, 25, 51]. Additionally, it has been shown in another study that Oct4 was highly expressed between 10 and 12 weeks of gestation [16]. It is thus, plausible that the expression of those pluripotent markers especially Oct4 may positively regulate the expression of other factors such as survivin, that is required for cell proliferation [52], and thus favors epigenetic mechanism(s) underlying the maintenance of pluripotency [53]. The gestation weeks, in which the expression of Oct4 was reported to be highly varied between studies, may be due to donor variations and the employment of different isolation techniques that confer the differential gene expression [54]. Besides that, it can also be inferred that the window of expression of Oct4 in AFSCs could be wider, which in this case, is between 10 and 20 gestation weeks, considering all those reported studies. However, samples harvested from 18-22 gestation weeks have been found to show lower level of Oct4 expression [51, 55]. This contradicts with the study done by Bai *et al.*, where AFSCs from AF samples of 16-22 gestation weeks demonstrated the retention of high stem cell potential. Particularly, c-Kit+ AFSCs showed significantly higher expression levels of key pluripotency factors; Oct4, Nanog and Sox2 than those AFSCs that are negative for c-kit, indicating that c-Kit+ cells possess higher pluripotency capacity [56]. This can again be attributed to the variations in samples donor and the isolation techniques used [54]. It is noteworthy that AF-derived cells from later gestational stage generally have lower proliferation rate than those from early stages, whereby the type of cells present in AF would be enriched displaying progenitor- or organ-specific markers. Taken together, the level of Oct4 expression most likely is one of the key determining factor which confers higher proliferative and regenerative capacity in these cells isolated throughout various gestational weeks. However, it is important to note that the Oct4 expression is not the sole determinant of pluripotency, it merely signifies that AFSCs have characteristics very close to embryonic/pluripotent cells and have better plasticity than adult stem cells including mesenchymal stem cells. Considering the fact that some studies showed the presence of other key pluripotent transcription factors, which clearly suggests the heterogenous pluripotent-like characteristics, thus it is plausible to make a remark that these cells may have higher plasticity than the adult stem cells. From a regenerative medicine perspective, it may be imperative to enhance the therapeutic potential of AFSCs from later gestational stage, a more optimized culture condition that could foster and sustain the pluripotent network, and the relevant machinery would be important for a preferable culture strategy for assessing their efficacious therapeutic applications.

3.2. Preconditioning and Microenvironmental Factors Confer Higher Regenerative Potential

Other than gestation week, pluripotency level of stem cells is largely influenced by the component from microniche such as growth factors, cell-cell contact and extracellular matrix (ECM), which would likely determine the cell fate to either remain pluripotent or multipotent or differentiate into a specific lineage [57]. However, there has not been much investigation on the influence of *in vivo* and *in vitro* microenvironment on AFSC proliferation, and most importantly, regulation of pluripotent marker expression. Uncultured AFSCs were reported to express Oct4, the stem cell factors vimentin and alkaline phosphatase (AP) [24]. The authors did not test the expression of other pluripotent markers in these AFSCs. Thus, checking the uncultured AFSC for pluripotent/hESC marker expression would give a better understanding on where these cells stand in the hierarchy of pluripotency and the influence of culture media on the enhancement of pluripotency characteristics when they are cultured and expanded for potential stem cell therapy application. Soluble growth factors in culture medium play a significant and direct role in activating signaling pathways that are crucial for conferring pluripotency in AFSCs. The variations in the pluripotent marker expression in AFSCs as reported in previous studies could stem from different isolation and culture techniques employed [54]. Thus, understanding the possible influence of culture media on the regulation of pluripotency (adaptation to culture) could help in establishing an optimized culture condition for further downstream application. Currently, the commonly used culture media for AFSCs is Chang medium as described earlier [28]. The second trimester samples cultured in Chang media and selected for c-Kit expression thereafter, were found to express Oct4, SSEA4 and Tra-1-60. This raises the question of whether the c-Kit+ cells inherently express all the three pluripotent

Table 3. Pluripotent and cell surface marker expression by AFS cells.

Cell Type	Isolation Techniques	Marker Expression	Differentiation Studies	Reference
1st trimester c-kit+	10-12th week of gestation, first selected based on plastic adherence, subsequent selected for c-kit, expanded on matrigel in Stemedia nutristem XF/FF	Pluripotent markers: Oct4, Nanog, Sox2, SSEA4, Klf4, c-myc, ALP, weakly express SSEA3, Tra-1-60, Tra-1-81 MSC markers: CD73, CD44, CD105, fibronectin, laminin PGC: C-kit, T, FGF8, Sox17, STELLA, DAZL, NANOS, VASA, SSEA1, FRAGILIS, PUM2)	Endoderm, ectoderm, mesoderm (Hepatic, neuronal, oligodendrocyte, adipogenic, chondrogenic, osteogenic)	Moschidou *et al*., 2012
2nd trimester	14th week of gestation, not cultured	Oct4, Vimentin, Alkaline phosphatase	NT	Prusa *et al*., 2003
	15-20th week of gestation, cultured in Chang medium(α-MEM, 20% Chang B, 2% Chang C, L-Glutamine, 20% ES-FBS/ Amniomax II/DMEM with 10% FBS	Pluripotent markers: Oct4, C-kit MSC markers: CD34, CD90, NCAM, FGF5, E-cadherin, FLK1 Germ layer: Brachyury, Tal-1, CXCR4, Sox17, GSC, AFP, progenitor (CD24-OB cadherin, CEBPG, GDNF, TTF-1, NKX2.5, PDX1)	NT	Da Sacco *et al*., 2010
	16-21st week of gestation, cells grown in Amniomed, supernatant harvested, subsequently cultured in Amniomed/DMEM with 20% FCS, or M199 medium with 10% FCS, ECGF	Pluripotent markers: Oct4, Rex-1, Runc-1, ABCG2. MSC- osteocalcin, PPARgamma2, GAP43, NSE, Nestin, MAP2, GFAP and beta tubulin III	Myogenic, adipogenic, neuro-glial, osteogenic	Bossolasco *et al*., 2006
	18-22 week of gestation, α-MEM medium, 15% FBS	Strong c-myc, moderate rex1, klf4, trace amt Oct4a. oct4b, nanog, sox2 (not detected in IF)	NT	Li *et al*., 2009
	Nutrient mixture Ham's F10 medium, 10% FCS	CD133, nestin, neurofilament, P75	Neurogenic	Prusa *et al*., 2004
	18th- 22nd week, resuspended in amniomax II complete medium	C-myc, klf4, trace amount of Nanog	Neural, cartilaginous tissue, glandular tissue	Li *et al*., 2013
2nd trimester c-kit+	Gestation week- NA AFS grown in α-MEM, 15% ES-FBS, 1% glutamine, 18% Chang B, 2% Chang C	Pluripotent markers: 90% Oct4 positive, SSEA4, Tra-1-60, MSC-CD29, CD44, CD73, CD90, CD105 MHC class I	adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages	De Coppi *et al*., 2007
	15-18th week of gestation, DMEM high glucose, with 10% FBS. After 72, cells selected for c-kit + and replated on D10/stemedia nutristem on matrigel coated plate (passage 5-10)	D10: Oct4, SSEA4, c-myc, low/null nanog, klf4, sox2 Stemedia nutristem:upregulation of Oct4, Sox2, klf4, c-myc	NT	Moschidou *et al*., 2013 (b)
	16-18th week of gestation, cultured in DMEM, subcultured in Stemedia Nutristem XF/FF	Oct4, SSEA4, CD29, CD44, CD73, CD90, CD105	NT	Moschidou *et al*., 2013 (a)
	Gestation week-NA α-MEM with 18% Chang B 15% ES-FBS, 2% Chang C	c-kit, CD44, CD90, CD105, N-cadherin	NT	Skardal *et al*, 2013
	Gestation week-NA α-MEM with 18% Chang B 15% FBS, 2% Chang C	NT	Osteogenic differentiation	Sun *et al*., 2010
	16th – 31st week of gestation. A-MEM, 18% Chang B, 2% Chang C, 2% ES-FBS	Oct4, Sox2, Nanog	Adipogenic, osteogenic, myogenic	Bai *et al*., 2012

MSC- mesenchymal stem cells, PGC- primordial germ cells, NT- not tested.

markers or it is due to culture adaptation. If the culture condition could, in part, determine the cell fate, then culturing AFSCs in hESC medium could possibly enhance the pluripotency level of the cells. Moschidou *et al*. (2013) showed interesting results of alteration in pluripotent markers expression when the culture medium of c-kit+ cells was switched from MSC medium to human ESC medium (Stemedia Nutristem with matrigel coated plate) [58]. C-kit+ cells cultured in MSC medium (DMEM with 10% FBS) expressed Oct4, c-Myc and SSEA4, and showed null or low levels of Nanog, Klf4, Sox2, Tra-1-60 and Tra-1-81. However, when the cells were sub-cultured in hESC medium with matrigel coating, then the cells showed up-regulation in Oct4, c-Myc and SSEA4 expression and also expressed Sox2 and Klf4. Furthermore, cells cultured in hESC medium were smaller in size and showed higher cell division kinetics and lower doubling time (22.8 + 1.9h) when compared to cells grown in MSC medium (33.2 + 2.5h). It is therefore, speculated that pluripotent gene expression is reversibly activated and may be coupled with the selection of a particular population to be expanded upon culturing in different medium. This remains to be further investigated, thus understanding of the culture condition-mediated changes in the pluripotency status of AFSCs would allow to obtain a more homogenous cell population that would be more suitable for efficient differentiation to generate high quality pool of cells for cell therapy. Further studies are needed to identify the variations in pluripotent marker expression in uncultured and cultured AFSCs, in an effort to know the exact effect of culture medium on the modulation of AFSCs growth and pluripotency status.

It has been previously reported that the therapeutic effect of pre-expanded AFSCs varied considerably compared to freshly isolated cells [59, 60]. This has been speculated to be in part due to epigenetic modifications, which can significantly hamper the self-renewal and differentiation capacity of the cells, as well as changes in the induction of differentiation process [50]. As a result, these changes could potentially affect the survival of the cells expanded *in vitro* and possibly reduce the time frame where cellular senescence is induced upon the long-term passaging. These statements further emphasize the importance of optimizing culture conditions, as they play a significant role in recapitulating the niche where stem cells reside *in vivo*, thus they are among the crucial factors affecting the growth and survival of cells in culture.

On a different note, Martinelli *et al*. cultured AFSCs in media supplemented with either the conventional Foetal Bovine Serum (FBS) or human platelet lysate (Lyset), with the purpose of elucidating the effect of these two components on the beneficial characteristics of AFSCs [61]. While no significant differences were observed between the two culture conditions, the findings can be important in corroborating the use of xeno-free medium, as an alternative to FBS-supplemented medium for expansion of cells intended for cell-based therapy. As reported in the study, AFSCs expanded the presence of Lyset maintaining the phenotypic characteristics compared to those cultured in FBS-supplemented medium [61], showing the expression of Oct4 and SSEA-4 the absence of CD45 andHLA-DR [62]. More importantly, the non-xenogenic Lyset supplementation did not compromise the proliferative, multi-lineage differentiation and immunomodulatory potential of AFSCs, and was not tumorigenic [61]. Additionally, analysis of the secretome of AFSCs cultured in both FBS- and Lyset-supplemented medium revealed the presence of paracrine factors such as IL-6, IL-8, macrophage migration inhibitory factor (MIF) and GRO-α, all of which are factors essential for wound repair and tissue regeneration. With regards to the findings discussed above, it can well be inferred that a humanized culture system can provide comparable outcomes, especially in terms of cell quality, as typical culture condition with animal-derived reagents. These cells expanded in serum-free media are also relatively safer and more GMP-compliant, thus it can be a better cell source for clinical application. The promising results with the use of serum-free culture condition can serve as the basis to encourage in performing the functional studies of AFSCs grown using humanized culture system, with the presumption that the therapeutic effects of AFSCs would remain optimal.

3.3. Reprogramming of AFSCs into Functional Pluripotent Cells via Genetic and Epigenetic Modifications

AFSCs are broadly classified as multipotent and are considered to possess potency between pluripotent hESCs and multipotent adult stem cells. Thus, priming AFSCs to a more primitive state with higher potency would not only be easier than somatic cells or adult stem cells, but it will also increase the inherent value of these cells in regenerative medicine. The advent of induced pluripotent cells (iPSs) in 2006 by Takahashi and Yamanaka demonstrated that with just a few transcription factors, committed adult cells could be reverted back to embryonic state [63]. Pluripotency was initially induced in mouse fibroblast cells by the overexpression of specific transcription factors, Oct4, Sox2, Klf4 and c-myc. The induction of pluripotency was first achieved in human somatic cells employing combinations of retroviral or lentiviral vectors encoding either Oct4, Sox2, Klf4 and c-myc or Oct4, Sox2, Nanog and Lin28, respectively. Since then, induced pluripotent stem cells (iPSCs) of different somatic origins, from healthy and diseased individuals have been generated using various techniques [64].

The efficient generation of iPSCs is dependent on three factors, i) choice of factors for reprogramming, ii) method of delivery and iii) type of target cells [65]. Cell types can significantly influence reprogramming efficiency. Generally, it has been identified that pluripotency can be conferred by strategies including reprogramming via genetic or epigenetic modification and by supplementing chemical/small molecules which are summarized in Table **4**.

The first study to generate iPSCs from AFSCs used the four Yamanaka factors [51]. This study reported generation of eight iPSC lines from AFSCs and reported the ES-like colonies developing six days after infection. The efficiency of reprogramming was also reported to be higher than the human foreskin fibroblast cells, indicating that the reprogramming efficiency appeared to be dependent on the state of potency of the cells. Similarly, Wolfrum *et al*. (2010) found that transfection of the four Yamanaka factors induced efficient generation of iPSCs from AFSC [64]. Human AFSCs were found to be reprogrammed in shorter time course with higher efficiency than other cell types, especially

Table 4. Reprogramming factors and strategies used in conferring pluripotent and stemness status.

Reprogramming Factors	Mechanism	Pluripotent Markers	Differentiation *In vitro*/*In vivo*	Pluripotent Induction (days)	Repro-gramming Efficiency	Reference
Oct4/Sox2/Klf4/c-Myc	Retroviral transfection	AP, Oct4, Sox2, SSEA4, Tra-1-60, Tra-1-81	NT	6 days	~1.525%	Li *et al*., 2009
Oct4/Sox2/Klf4/c-Myc	Retroviral transfection	Oct4, Sox2, Nanog, SSEA4, Tra-1-60, Tra-1-81, AP	All three germ layer *in vitro*, teratoma *in vivo*, Trophoblast	7 days	Not available	Wolfrum *et al*., 2010
OCT3/4, SOX2, KLF4, and c-MYC	Retroviral transfection	AP, OCT 3/4, SOX2, SSEA4, and NANOG	3 germ layers, EBs and teratomas	7 days	Not available	(Galende *et al*, 2010)
OCT4, SOX2, KLF4 and cMYC.	Lentiviral transfection	OCT4, SOX2, NANOG, SSEA-3, SSEA-4, TRA-1–60 and TRA-1–81	3 germ layers, EB, neu-ronal progenitor cells	7 days	Not available	(Lu *et al*, 2013)
OCT4, SOX2, KLF4 and cMYC.	Polycistronic vector	SSEA3, SSEA4, Tra-1-60, and Tra-1-81	Teratoma formation and cardiomyocyte diferentiation	10 days	0.4%	(Ge *et al*, 2012)
Oct4/Sox2/Klf4/c-Myc, VPA, Vitamin C	Lentivirus transduction, epige-netic modifiers	AP, Nanog, Rex1, Oct4, TRA-1–60, TRA-1–80, SSEA-3, and SSEA-4.	EBs, teratomas (3 germ layers) and functional motor neurons	13-15 days	0.1%	(Cai *et al*, 2010)
OCT4, SOX2, KLF4, and cMYC.	Non-integrating Sendai viral vectors	NANOG, OCT4, SOX2, SSEA3, SSEA4, TRA-1-60, and TRA-1-81	3 germ layers, EB's and differentiation to neural progenitor *in vitro*, mature neurons *in vivo*	14-21 days	0.01 % - 0.05%	(Jiang *et al*, 2014)
OCT4/SOX2/NANOG	Lentivirus transfection	AP, OCT4, SOX2, NANOG, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81	All three germ layer and teratomas	5 days	0.1%	(Zhao *et al*, 2010)
Oct4/Klf4	Retroviral transfection	Oct4, Nanog, Tra-1-60	Neural, cartilaginous tissue, glandular tissue	9 days	0.01 %	Li *et al*., 2013
OCT4 and KLF4	Retroviral transfection	AP, OCT4, NANOG and TRA-1-60	EB's, 3 germ layers, cardiomyocyte differentia-tion *in vitro*, and teratomas *in vivo*	20 days	0.01%	(Li *et al*, 2013)
Oct4	Retroviral transfection	AP, Nanog, Oct4, Sox2, and Rex1	Multilineage blood cells	4 days	Not available	(Liu *et al*, 2012 Liu *et al*, 2012)
Valproic acid (VPA)	Histone deacetylase inhibitors (epige-netic modifiers)	Oct4, Nanog, Sox2, Klf4, c-myc, SSEA3, SSEA4, Tra-1-81, Tra-1-81, ALP	All three germ layer *in vitro*, teratoma *in vivo*	5 days	Not available	Moschidou *et al*., 2012
Valproic acid (VPA)	Histone deacetylase inhibitors (epige-netic modifiers)	Oct4, Klf4, c-myc, Sox2, Nanog, SSEA3, SSEA4, Tra-1-60, Tra-1-81	All three germ layer, teratoma *in vivo*	5 days	Not available	Moschidou *et al*., 2013b

somatic cells. Using AFSCs, Li *et al.*, were able to show that they can be reprogrammed back to iPSCs via ectopic expression of the same four factors in less than a week [55]. Besides rapid reprogramming, the cells were efficiently converted to iPSCs at a frequency of 1.525%, 100-fold more efficient than previously reported on the fibroblast cells. Similarly, other research teams used OKSM factors to induce pluripotent AFSCs via retroviral transfection within 7 days, and the resultant cells were also able to differentiate into the three germ layers, which are a hallmark of iPSCs. In another recent study, it was reported that only two factors (Oct4 and Klf4) are needed to reprogram the human AFSCs [55]. Furthermore, obviating the requirement for c-Myc reduces the risk of inducing tumorigenicity [55]. Taken together, AFSCs are at a precursor state and are therefore, more receptive to being converted to a pluripotent state. The ability of AFSCs to be reprogrammed with higher efficiency may also be due to the lack of epigenetic restrictions or bivalent characteristics in these cells as compared to adult somatic and stem cells.

In search of potential small molecules that could enhance the reprogramming efficiency, Cai *et al.* found that OKSM with a combination of vitamin c and valproic acid (VPA) drastically increased the generation of human iPSCs. This leads to the question that whether AFSCs are more susceptible to reprogramming via integrating or non-integrating methods [66]. Besides that, it is also worth noting that the heterogeneous composition of AFSCs may play a role in reprogramming efficiency. The variability in reprogramming efficiency is largely due to the heterogenic nature of AFSCs. Characterization of these cells has shown that cells within subpopulations express embryonic markers, whereas other

subpopulations express adult stem cell markers or both. Studies done on AFSCs that was of a homogenous population (eg: surface marker c-kit or CD117 positive) produced more efficiently reprogrammed cells [67]. However, further in-depth studies are needed for assessing the complete characterisation of AFSCs in order to realise their use in clinical medicine.

The ectopic expression of transcription factors (even it is reduced to fewer factors) may limit the clinical use of AFSC-derived iPSCs due to the risk of random integration and viral-induced tumorigenicity and has also been proven to be of low efficiency. Thus, an alternative approach to generate iPSCs that is transgene-free based has been investigated using small molecules and epigenetic modifiers. In 2012, a study reported on the generation of transgene-free AFSC-iPSC using the epigenetic modifier, valproic acid (VPA). VPA, a histone deacetylase inhibitor (HDACi), induces conversion of first trimester AFSCs to a more primitive state that has similar molecular and cellular characteristics as hESCs. VPA supplementation was shown to upregulate pluripotent gene expression of Oct4, Sox2, Nanog, Klf4, c-Myc and causes homogenous expression of SSEA3, SSEA4, Tra-1-60, Tra-1-81 and ALP [16]. The same group studied the possibility of using VPA on mid-trimester AFS cells to achieve the same outcome. They found that cells grown in hESC medium supplemented with VPA for 5 days showed remarkable upregulation of pluripotent markers Oct4, Sox2, c-myc, Klf4, Nanog, SSEA3, SSEA4, Tra-1-60 and Tra-1-81 compared to the untreated cells. It is interesting to see that Lin28, which involves in epigenetic switch to confer the pluripotency in transgene-based method, could possibly be dispensable, as VPA could achieve the reprogramming by employing the epigenetic mechanism. In this case, the native Oct4 expression may be sufficient to activate the pluripotency network (SOX2, Nanog) in cooperation with VPA's action on epigenetic landscape in AFSCs.

The above studies on the development of AFSC-iPSC show distinctive advantage over iPSC generated from other cell sources like fibroblast cells. Firstly, AFSCs can be routinely harvested from amniocentesis samples. This cell source also carries fewer environmental-induced mutations and is genetically stable. Secondly, reprogramming of these AFSCs is comparatively easier than somatic cells, as only two factors are needed for the generation of iPSCs. Since the cells can be reverted to pluripotent status with only exogenous supplementation of Oct4/Klf4, it does not require the use of c-Myc which, in turn, reduces the risk of tumorigenicity making it more operational for clinical medicine [25]. Moreover, since only two factors (Oct4/Klf4) are needed for genetic modification, it is possible to substitute one or more factors with small molecules (VPA) that activate Oct4/Klf4 as discussed above.

4. APPLICATION OF hAFSCs IN THE AMELIORATION OF HUMAN PATHOLOGIES AND DISEASES

As AFSCs possess higher proliferation and differentiation plasticity than adult stem cells, it is sufficiently justifiable to say that their usage in transplantation medicine would offer higher rate of success. Additionally, since AFSCs are non-tumorigenic and do not beget ethical controversies, as do ESCs, they are said to be the optimal stem cell source for regenerative medicine.

With much heated ongoing interest and investigation on AFSCs as the new leading therapeutic cell source, functional studies that showcased the beneficial properties of AFSCs in the treatment of human pathologies are also presently available. Pertaining to the regenerative potential of AFSCs, it has been thought to be an excellent cell source for skeletal diseases, such as bone defects that are characterized by compromised tissue regeneration [68]. Supporting this notion, AFSCs have been tested successfully for their ability to produce mineralized matrix and bone tissues *in vivo* [28, 29, 69-72]. Intriguingly, osteogenic differentiation of AFSCs can take place in a short period of time, and the resulting osteogenic progenitors after 30 days have been shown to express the complete set of osteogenic markers, consisting of COL1, OCN, ONC, OPN, OPG, BSP and Runx2 [73]. This finding strongly supported the safety and reliability of utilizing AFSC-producing osteocytes for therapeutic purposes. As a promising candidate in the realms of bone regeneration, AFSCs have been shown to be a viable cell source that can be employed in postnatal sternal repair [74]. These stems from the observation that engineered AFSC-derived bone grafts contributed to bone formation and chest closure, thereby promoting chest wall reconstruction.

Attributed to their capacity to also differentiate along the neural lineage [28, 75-77], AFSCs are regarded as potential cell source for clinical treatment of brain disorders. The latter title for AFSCs also owes it to their immunomodulatory and anti-inflammatory property, as well as their ability to induce angiogenesis and neurogenesis [78]. Experimental evidence has been provided on the positive outcome with the use of AFSCs for focal cerebral ischemia, where mitigation in brain damage with improved behavioral functions was present [79]. Besides that, AFSC transplantation is said to be especially useful for acute myocardiac infarction with cardiovascular etiology, as these cells also contribute to improved cardiac function [80]. Furthermore, it has been observed in several studies that AFS transplantation improved the therapeutic outcome of ischemic stroke by reducing inflammatory response in the affected region [75, 77, 81, 82]. Therefore, AFSCs as the cell source for transplantation can be particularly beneficial, in terms of functional recovery, for patients with stroke and other neurological disorders characterized by neuroinflammation [83].

Interestingly, it has been recently reported that AFSC secretome contains key molecules that mediate tissue repair and regeneration in muscle damage, possibly through their observed ability to stimulate stem cell proliferation and migration, induce angiogenesis, as well as their anti-inflammatory and anti-senescence effect [84]. Besides this, in an experimental model of colitis, secreted molecules (in conditioned media) from AFSCs have been demonstrated to ameliorate the condition through reducing inflammatory response, as evidenced through the increased expression of IL-10, as well as the decrease in TNFα and IL-1β expression [85]. Similarly, AFSCs have been reported to promote neovascularization through releasing a set of angiogenic factors such as MMP-9, MCP-1, endothelin-1, angiogenin and TIMP-1 [86]. This finding further fortifies the promising

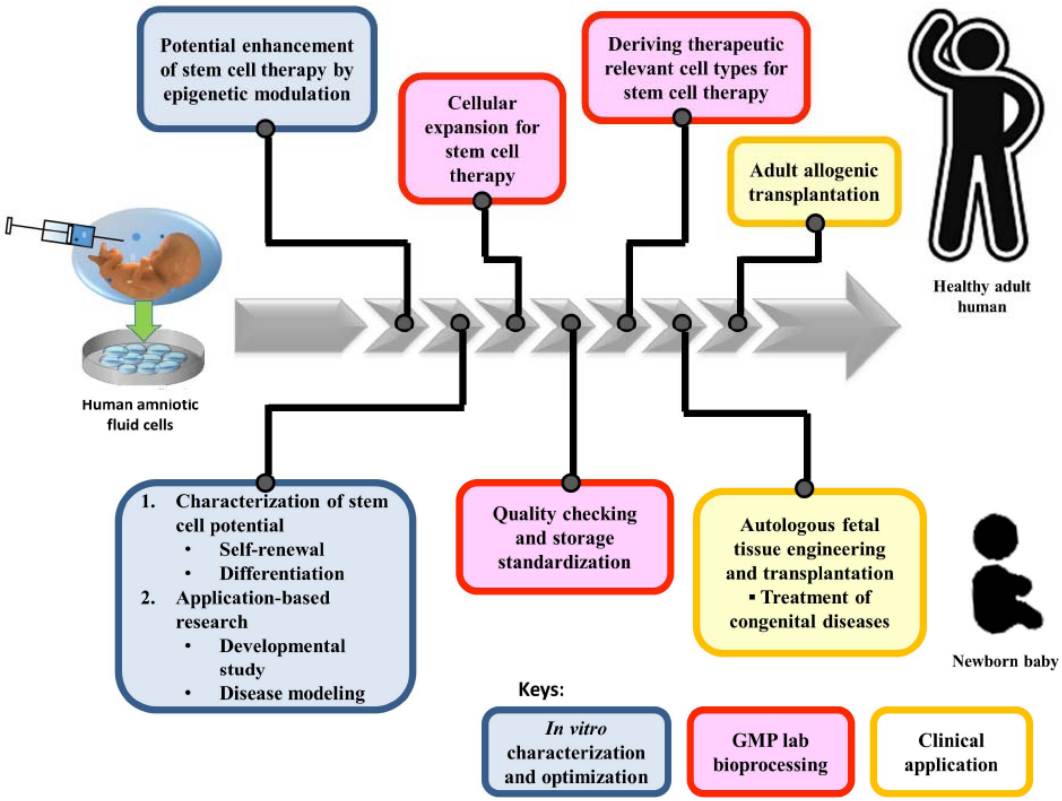

Fig. (1). Roadmap of AFS cells from bench to bedside application. The work flow to develop hAFSC based therapy consist of 3 therapeutic development packages; i) *in vitro* characterization and optimization, ii) bioprocessing of stem cells in Good Manufacturing Practice (GMP)-certified laboratory and iii) development of cell therapy procedures and clinical trials.

therapeutic benefit and outcome with the use of AFSCs for clinical, medicine related to tissue repair and regeneration.

5. FUTURE PERSPECTIVES

Based on all the facts documenting that hAFSCs possess the highest potential among other stem cell types, exploitation of these cells in regenerative medicine can be very promising. In this regard, it has been proposed that step-wise development of hAFSC-based cell therapy (Fig. **1**) can potentially improve the therapeutic outcome and improved efficacy in treating many degenerative and genetic diseases in both fetal and adult medicine. As continuous expansion of cells in culture is associated with the production of aged cells with decreased potential for clinical medicine; however, this problem can be tackled through epigenetically modulating the biology of these expanded cells, so that they can be rejuvenated to a higher clinical potential prior to being exploited for therapeutic purposes.

It is equally important to characterize the potential of hAFSs, by assessing the prominent features of stem cells – the self-renewal and differentiation capacity prior to their use for transplantation. For application-based research, developmental study and disease model testing can be done to further validate the potential of hAFSs in regenerative medicine. After the expansion process, hAFSs needs to be bioprocessed, to ensure the safety profile of these cells which must be in compliance with the GMP standard. This step also aids in deriving cells that are therapeutically relevant, and perfect to be used in stem cell-based therapy [87]. There are two ways in which hAFSs can be transplanted into the human body, either through the allogeneic or in autologous manner. The autologous transplantation of hAFSs is more suited for infants who are prenatally diagnosed with birth defects, while allogeneic transplantation can be adopted for the treatment of a range of human diseases, without the risk of immune rejection. In short, exploitation of hAFSs for clinical application to treat human pathologies in order to improve the therapeutic benefit and improved outcome of stem cell-based therapy which, in turn, will improve patients; outcome suffering from diseases that are degenerative in nature.

As discussed previously in regard to the fact that AFSC's secretome also possesses therapeutic potential that can be exploited for clinical treatment of diseases whereby, a comparison can be made between secretome from different stem cell sources, in terms of the proliferative and differentiation capacity. This can be an additional layer of characterisation of AFSCs, as well as validation of their greater therapeutic potential compared to other stem cell types. Furthermore, analysis of the constituents comprising AFSC's secretome can be done to potentially elucidate secreted molecules from AFSCs that may play a significant role in dictating the high therapeutic potential of these cells. Supporting this notion, it has recently been reported that AFSC-derived extracellular vesicles (EVs), which are released as part of the secretome, showed both paracrine and regenerative potential [88]. Interestingly, microRNA (miRNA) profiling revealed that these EVs released miRNAs that are essential for angiogenesis such as miR-126 [89-91] and miR-210 [92, 93], proliferation

including miR-199a-3p [94] and miR-210 [95], as well as miR-21 that activates the ERK1/2-Stat3 signaling pathway conferring pro-survival effect [96]. Thus, elucidation of the secretory profile of AFSCs can further empower our understanding of their characteristics and the associated secreted molecules, thereby aiding in effective cell preparation that would ideally preserve the potential of AFSCs pre-transplantation melieu. In extension of the latter notion, pertaining to the fact that AFSC's secretome comprises of key regulators of tissue regeneration, it is of paramount importance to ensure the production of secretome that is biologically active and without the presence of exogenous molecules [84]. This warrants the need to devise enrichment protocol that would potentially enhance the production of EVs that release molecules conferring high regenerative potential, such as through hypoxic preconditioning as reported recently [88].

Besides exploring the therapeutic characteristics of AFSC's secretome, another practical approach would be having AFSCs to be tested and validated for their regenerative potential in pre-clinical animal models. The resulting positive outcome would allow AFSCs to be proposed as the cell source in clinical trials to treat human diseases, which would be a milestone in the advancement of stem cell-based therapy. Pertaining to the fact that AFSCs are also potential cell source for *in utero* transplantation to treat congenital disorders [97, 98], their therapeutic characteristics could be exploited in the modelling of human genetic diseases for further studies. The latter is especially important, because AFSCs represent a well-suited alternative stem cell source, obviating the need for iPSC generation to discover the molecular biological changes associated with genetic disorders [99, 100]. Besides that, the imperative need is to translate AFSCs from bench to clinic stems from the demonstration of their anti-cancer activity *in vitro* [101-103]. Further exploration of their anti-cancer effect in preclinical animal models would pave the road to ultimately utilize them in cancer treatment, which may possibly and plausibly replace the need for chemotherapy in the future that is often associated with the undesirable side effects, causing drug resistance and toxicities to normal cells [104].

CONCLUSION

AFSCs provide several advantages over other cell types in terms of their greater therapeutic potential by expressing partial pluripotent markers in addition to multipotency markers, especially because much of the work has been focused on deriving therapeutically relevant cell types representing all of the three germ layers. Research on exploring the potential applications of AFSCs in regenerative medicine and disease modeling is still at its infancy. This is in part due to the lack of understanding of their exact pluripotency and microenvironmental factors that may influence their self-renewal and differentiation capacity. Thus, there is still much investigation to be done in order to fully understand this novel stem cell population. The *in vitro* expansion and differentiation capacity and pluripotency levels under various influencing factors like culture media, are yet to be fully understood, and there is a demand for exploring more interesting areas on the biology of AFSCs. For instance, it would be interesting to examine how pluripotency can be conferred to these cells via optimization of ECM (mediated by E-cadherin molecule) and the possibility of using nuclear transfer. Other than extracellular niche factor, the epigenetics, signaling pathways involved and the differentiation potential of these cells are worth exploring. This would provide a better understanding of AFSCs and will provide a promising cell source for cell therapy targeting congenital diseases which could be treated before birth or in the neonatal period. Moreover, the overall usefulness of AFSCs for achieving better therapeutic outcome in the arena of adult clinical medicine is very promising which would become a great success story in modern medicine.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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